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Exposing Lettuce Plants to Cyanobacteria in a Closed Hydroponics System to Reduce Cyanobacterial Growth and Production

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Abstract: Sandusky Bay is largely populated by cyanobacterial algal blooms, mainly formed by *Planktothrix*. Fertilizers containing nitrogen and phosphorus run from agricultural lands into the bay, building up excess nutrients forming eutrophic waters. The *Planktothrix* feed off these nutrients and grow into algal blooms. To determine a potential solution to the growth of these blooms, I implemented a hydroponics system involving *Lactuca Sativa* for analysis of *Planktothrix* growth and productivity. Four different nutrient conditions were added to a *Planktothrix*-only solution and a solution growing *Planktothrix* with the lettuce in the hydroponics system. The four conditions consisted of no nutrient addition (control), base levels of nitrogen and phosphorus (NP), high nitrogen base phosphorus (HN), and high phosphorus base nitrogen (HP). My results concluded that when *Planktothrix* was exposed to HN and HP conditions, it grew substantially more when in an environment alone than when growing with lettuce. The rate of growth and cell counts were higher in those solutions of *Planktothrix*-only, suggesting a possible competitive relationship formed between the bacteria and the lettuce in which the lettuce out-competed the bacteria for nutrients. This analysis can conclude a possible solution to the algal blooms in Sandusky Bay where a change in agricultural systems may help mitigate *Planktothrix* growth. The implementation of a hydroponics system where eutrophic water is recycled back into the agricultural systems, or where cultivation of crops occurs right on the bay itself may lead to crops outcompeting bacteria for nutrients, leading to a decrease in algal blooms within the water.

Introduction

Sandusky Bay, an inlet located off the southern region of Lake Erie, has been threatened with harmful cyanobacteria for decades. Cyanobacteria are a form of bacteria that live in water and make up blue-green algae, often forming into blooms. If the cyanobacteria produce toxins in the water, the blooms are known as cyanobacterial harmful algal blooms, like those found in Sandusky Bay. The major harmful cyanobacteria that construct these blooms are *Planktothrix* and *Microcystis*. The toxins these bacteria produce are called microcystins, which can pollute waters and cause harm to living organisms. The algal blooms that populate Lake Erie consist of predominantly *Microcystis* over *Planktothrix*, however, in the surrounding bay areas, *Planktothrix* is more commonly found in the algal blooms. While Lake Erie blooms have been seen to fluctuate in size with changing seasons and weather conditions over the years, the blooms found in the Sandusky Bay are consistent in size patterns despite fluctuating weather conditions. (Davis T.W. et al., 2015). Studies have shown that algal blooms form and thrive from eutrophication of the water where nutrients, especially nitrogen and phosphorous, are added in excess amounts through agricultural runoffs into local rivers and streams that dump into the lake and surrounding bays. Lake Erie is especially susceptible to eutrophication due to its small size and relatively shallow depth, allowing nutrients to accumulate more rapidly. The cyanobacteria in the water then take up these nutrients to use for growth and productivity. Algal blooms can

block sunlight from entering through the water, decreasing oxygen production from aquatic plants, and leading to massive fish death (Salk et al., 2018). Algal blooms can be dangerous for the people living near the waters they infest as well as the organisms in the water column.

Efforts are continuously being made to solve the problem of these algal blooms. One potential solution is the application of hydroponics. Hydroponics is a system involving the growth of plants in a medium other than soil (for example water). This approach to growing plants utilizes the processes of recycling nutrients and water to conserve materials and money (Wongkiew et al., 2017). Plants, similar to cyanobacteria and other living organisms, utilize nutrients like nitrogen to grow and survive. In a hydroponics system, the nitrogen the plants obtain is introduced into the water manually through various fertilizers or nutrient mixes, or naturally through nutrient runoff from fertilizers. (Enduta et al., 2011). The cyanobacteria that form algal blooms also take in those same nutrients through nutrient runoff similar to a hydroponics system (Salk et al., 2018). If the plants in a hydroponics system and the cyanobacteria producing algal blooms are combined in one system with nutrient introductions, a competitive relationship may form.

This research project will be combining the mechanisms of hydroponics with the known life patterns of cyanobacterial harmful algal blooms. It will introduce the following question: Can lettuce plants outcompete cyanobacteria for nutrients when growing in the same water column? The results are anticipated to show a decrease in algal blooms and *Planktothrix* counts with the lettuce plant growth. If the plant roots can take up nutrients faster than the cyanobacteria, there will be a depletion of nutrients left for the cyanobacteria to live and thrive from, and their growth will be reduced. With enough time, the lettuce will outcompete the cyanobacteria. The null hypothesis of this experiment based on the research question is as follows: If growing lettuce is introduced to a culture of *Planktothrix*, the lettuce will not affect the growth or production of that bacteria.

Relevance: This research serves to replicate the waters of the Sandusky Bay, which are infested with cyanobacterial harmful algal blooms. The results of this experiment will provide information that may help to reverse the growth of those algal blooms and bring the water quality up. This study will utilize the parallels of nutrient importance between cyanobacteria and lettuce plants to try to discover a conservative way to clean the bay's water. A multidisciplinary approach including agriculture in aquaponics and microbiology cell counts can ultimately

provide a solution to a problem that Sandusky Bay has been suffering from for decades. Clean water results in a safe environment that can support the steady growth of fish, influencing both the environment and the economy positively. If the predicted outcomes are shown, these results can be used to create an integrated system that expands far beyond the laboratory and into the city of Sandusky and surrounding areas. Agriculture can evolve to include nutrient and water recycling in Ohio by using eutrophic waters in agricultural systems instead of introducing more nutrients and polluting the waters.

Methods and Materials

Figure 1: October 1: Lettuce/Planktothrix setup. Initial size of lettuce at introduction to beakers with nutrients and Planktothrix.

Figure 2: October 1: Initial Planktothrix setup with first nutrient addition.

Set up: This project was completed in the research lab on floor two of the Life Sciences building at Bowling Green State University. Twenty-four beakers were lined in columns of three beakers each on the solid countertops of the lab. The beakers were located near air valves, where airline tubing was connected from the valves to the beakers with bubblers suspended in the water to help with aeration. The beakers were properly labeled as either a control (C), nitrogen and phosphorous base (NP), high nitrogen (HN), or high phosphorus (HP), with three beakers for each scenario as replications. Two clamp grow lights with 75-Watt incandescent bulbs were suspended above the beakers, one shining light on the bacteria subjects and one shining on the lettuce/bacteria subjects. Saran wrap was used to cover each of the twenty-four beakers to mitigate any evaporation from the lights.

Lactuca Sativa, Burpee Black Seeded Simpson Lettuce, was planted in Lambert LM-GPS soil and cared for in the BGSU Greenhouse. After one month of growth, the lettuce was extracted from the soil, the roots were carefully rinsed to get rid of as much soil as possible, and the plants were put in the freshwater beakers. At the time of introduction to the beakers, the average dry weight of the lettuce was 0.44g. Then, 10 mL of the non-toxic *Planktothrix* culture obtained from the Davis Laboratory at BGSU was measured out and added to each of the twenty-four beakers giving an initial density of 80,986 filaments per mL per beaker.

Nutrient addition: Nature's Nectar Phosphorus 0-2-0 with guaranteed minimum analysis of available phosphate 2.0% was used to add phosphorus to the subjects that needed it. To create the base stock solution, 500 mL of water was mixed with 1.5 mL of the Nature's Nectar. 10 mL of this base stock were added to all beakers labeled HN and NP. For the beakers with high phosphorus, the recipe for the stock solution was doubled in terms of Nature's Nectar addition, so 3 mL of the nutrients were added to 500 mL of water, and then 10 mL of that solution were added to all beakers labeled HP. Nature's Nectar Nitrogen 5-0-0 with a guaranteed minimum analysis of total nitrogen 5.0% and 5.0% other water soluble nitrogen was used for the addition of nitrogen nutrients. To create the nitrogen stock solution, 500 mL of water was mixed with one mL of the nitrogen solute, and 10 mL of this stock was added to all beakers labeled HP and NP. For the subjects with high nitrogen, the nitrogen solute was doubled, and the solution was made by adding 2 mL of nitrogen into 500 mL of water, and 10 mL of this solution was added to all beakers labeled HN. All beakers labeled C received no added nutrients. After the second week of samples were collected, these solutions were made again and added to their respective beakers, however only 5 mL was added this time. These nutrients, along with the lettuce and *Planktothrix* were added to the beakers on October first and ran for one week before the first set of samples were collected.

Sample collection: Sample collection began on October eighth and samples were taken every week on Thursday until the fourth set of samples were collected on October twenty-ninth. Every week, the beakers were topped off with water to correct for the amount of water that had evaporated through the week, if any. Each of the water levels therefore were maintained at 250 mL. Before samples were collected, the water in each beaker was stirred enough to ensure that any bacteria were distributed evenly throughout the water. 5 mL of water was taken from each beaker and collected in a falcon tube. About one drop of Lugol's solution was added to each sample to preserve the bacteria, and the samples were placed in a drawer to block off all sunlight from affecting the function of the Lugol's solution. Using an inverted microscope, 15 μL of each sample were manually observed to count the filaments in that sample. Eight counts of each sample were taken. The average count per mL was then computed electronically based on the measurements of the slide the samples were placed on and the number of filaments counted. These averages for each beaker were recorded each week, and the averages of each category between the three replicates were averaged together to get one total count per scenario per week.

Results:

All densities are measured in cell counts/mL. All samples beginning with B represent those with only the *Planktothrix* culture, and those with an L represent the samples with lettuce and *Planktothrix*:

Table 1: Sample 1 Averages: Oct. 8th, 2020

BC	53,991	**LC**	34,708
BNP	38,565	**LNP**	26,995
BHN	42,421	**LHN**	34,708
BHP	46,278	**LHP**	15,426

Table 2: Sample 2 Averages: Oct. 15th, 2020

BC	3,856	**LC**	38,565
BNP	96,412	**LNP**	138,833
BHN	420,356	**LHN**	30,852
BHP	339,370	**LHP**	15,426

Table 3: Sample 3 Averages: Oct. 22nd, 2020

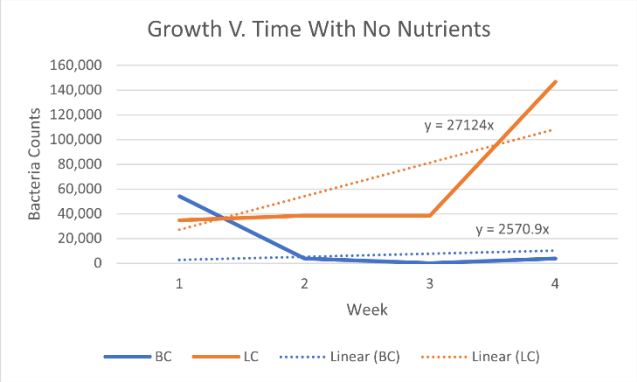
BC	0	**LC**	38,565
BNP	185,111	**LNP**	242,958
BHN	1,604,296	**LHN**	119,551
BHP	586,185	**LHP**	65,560

Table 4: Sample 4 Averages: Oct. 29th, 2020

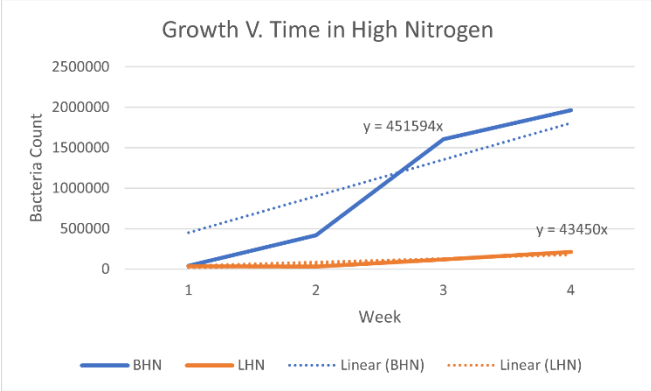
BC	3,856	**LC**	146,546
BNP	316,231	**LNP**	158,116
BHN	1,962,949	**LHN**	212,107
BHP	617,037	**LHP**	92,556

To determine whether the end result of *Planktothrix* counts were significantly different comparing B trials with L trials, the counts for each replicate of each trial from sample 4 was entered into R script. A constant 1 was added to each count to ensure that no counts were at zero. The log with base ten was taken of each count to stabilize the variances between data points. With the necessary assumptions now fulfilled, a two-way ANOVA test was run on the data. The ANOVA test determined that there was data within the counts that was significant, and a pairwise t-test was conducted to determine which data were significant with each other. The pairwise test concluded that the two variables that were significantly different from each other were BC and LC, with no other variables significant. This difference in counts between the control group with lettuce and without lettuce was the driving force behind the significance of the ANOVA test. If the control groups were taken out of consideration when analyzing the data, the ANOVA would have concluded insignificant overall (see Plot 1 and Plot 2 for comparison of results). Upon further analysis of the results, the averages over time between each paired trial was graphed to determine if rate of growth was changed when lettuce was introduced. The following results are graphed below (see Graph 1, Graph 2, Graph 3, and Graph 4 for results).

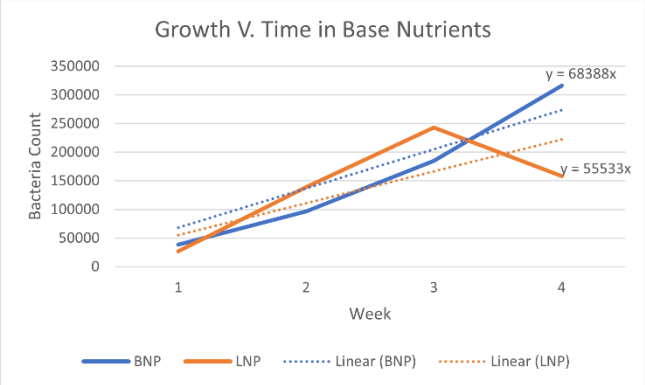
Graph 1: Growth Over Time Control



Graph 3: Growth Over Time HN



Graph 2: Growth Over Time NP



Graph 4: Growth Over Time HP

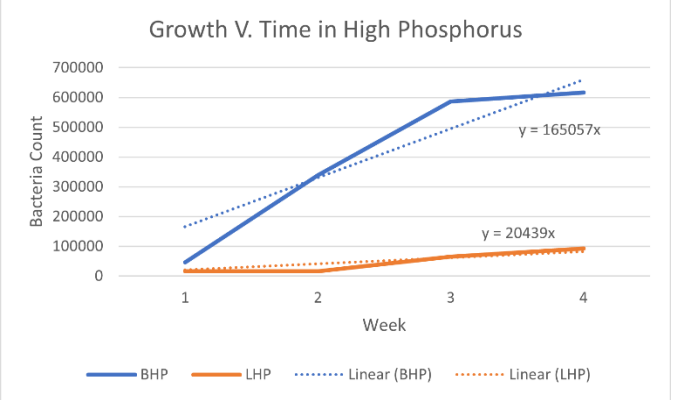
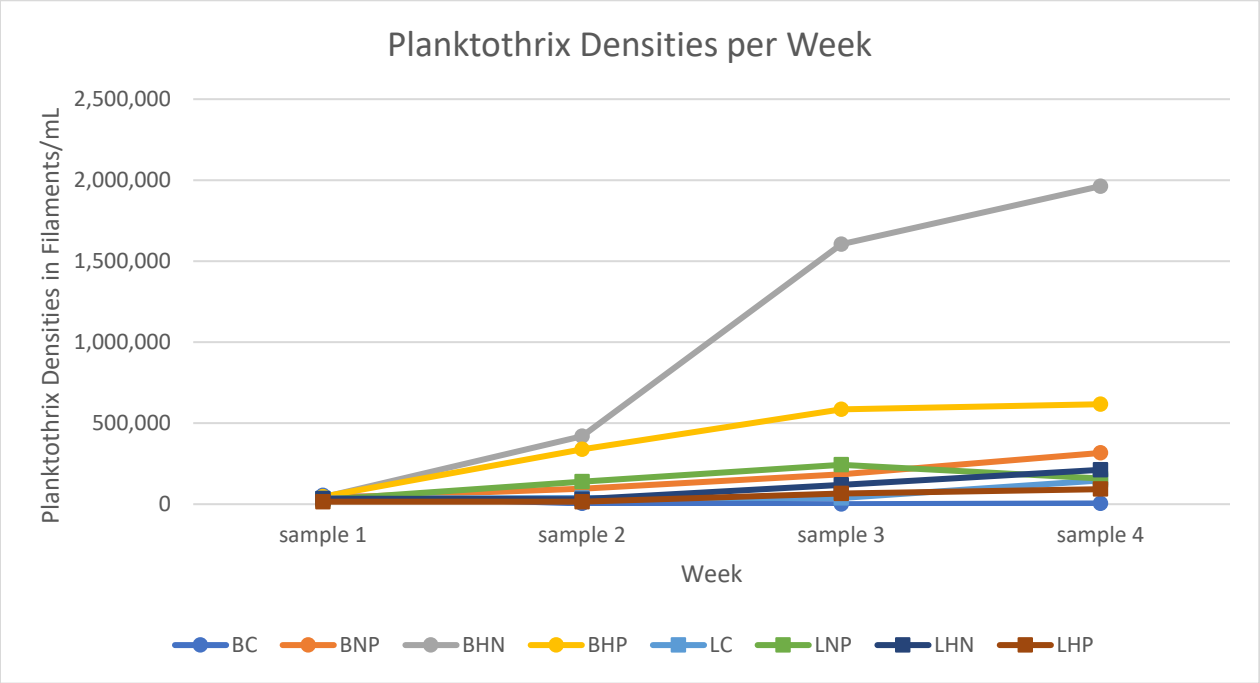


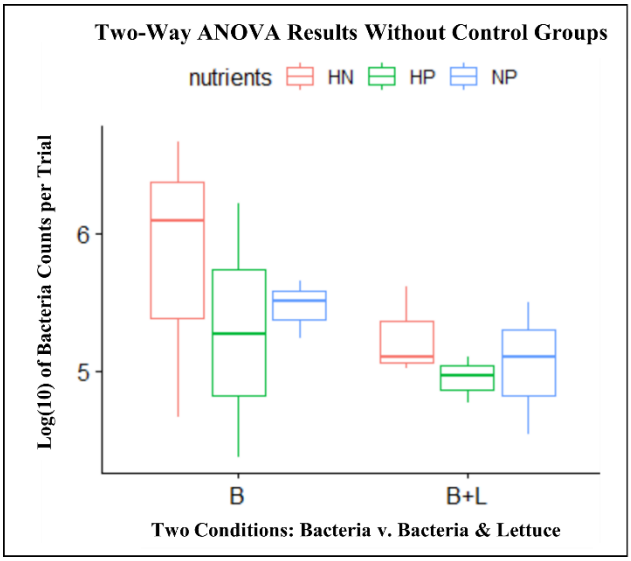
Table 5: Sample 4 Total Replicate Data:

BC1	0	**LC1**	231,389
BC2	11,569	**LC2**	57,847
BC3	0	**LC3**	150,403
BNP1	451,208	**LNP1**	127,264
BNP2	173,542	**LNP2**	34,708
BNP3	323,944	**LNP3**	312,375
BHN1	4,604,639	**LHN1**	404,931
BHN2	1,237,931	**LHN2**	104,125
BHN3	46,278	**LHN3**	127,264
BHP1	1,642,861	**LHP1**	92,556
BHP2	185,111	**LHP2**	127,264
BHP3	23,139	**LHP3**	57,847

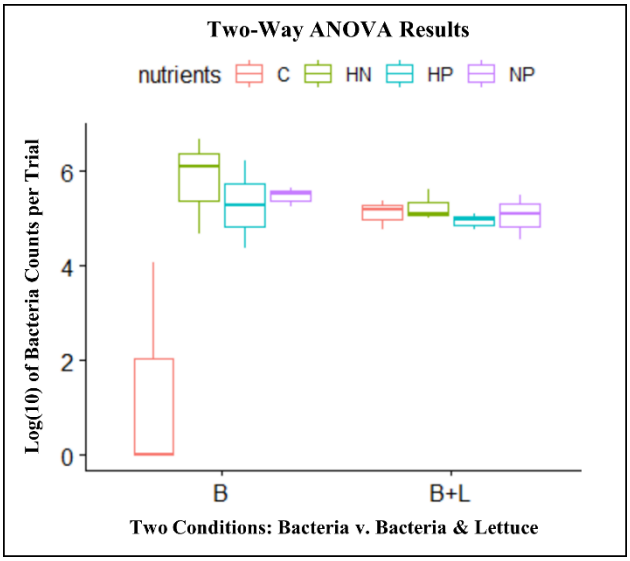
Graph 5: Total Average Densities Over Time



Plot 1: ANOVA Results Without Control



Plot 2: ANOVA Results With Control



Lettuce Productivity:

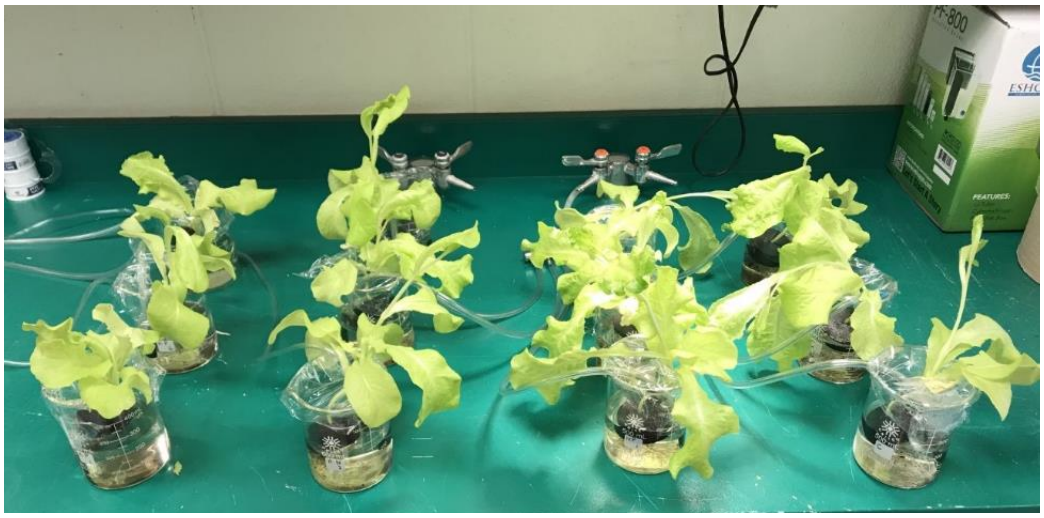


Figure 3: Week 1 lettuce progress. From left: LHP1-3, LHN1-3, LNP1-3, LC1-3. All visibly healthy and successful, LHP1-3 slightly smaller than the rest.

Figure 4: Week 2 lettuce progress. Starting from left: LHP1-3, LHN1-3, LNP1-3, LC1-3. Visible differences in productivity in plants, with LHP1-3 and LHN1-3 showing lower productivity.

Figure 5: Week 3 lettuce progress. Starting from left: LHP1-3, LHN1-3, LNP1-3, C1-3.

Figure 6: Week 4 lettuce progress. Starting from left: LHP1-3, LHN1-3, LNP1-3, LC1-3. LHP1-3 and LHN2-3 all completely decayed. LC1-3 and LNP1-3 alive but decreasing in productivity.

Discussion:

Based on the results of the ANOVA and pairwise t-test, the only significant difference in results was the controlled growth of bacteria in lettuce versus non-lettuce trials. The conclusion suggested that the samples with lettuce and *Planktothrix* with no nutrient addition provided significantly higher *Planktothrix* densities than the samples of bacteria in the control group without the addition of lettuce. All other trials were determined to be insignificantly different from each other, failing to reject the null hypothesis that if lettuce is introduced to a culture of *Planktothrix*, the lettuce will not affect bacteria growth and density. The pairwise test concluding that the control groups alone were significantly different was surprising to find, given that there was no nutrient addition in either trials so there was expected to be little to no growth of *Planktothrix* in either conditions. The elevated densities of *Planktothrix* in the trials with lettuce could have resulted from the addition of minute traces of nutrients from the roots of the lettuce, where nutrients from the roots were added to the water allowing the *Planktothrix* densities to climb. Because there would be no possible way for nutrients to be added to the control group without the lettuce, those densities stayed constant or went to zero over the four weeks, causing the two groups BC and LC to be significantly different. The results that no other densities between lettuce and non-lettuce groups was also surprising, especially within the high nitrogen trials. There was visibly more *Planktothrix* growth in the bacteria trials of HN than in the lettuce trials, and the counts of BHN1 were at 4.6 million cells/mL. Figure 7 displays this visible growth of *Planktothrix*. This was the highest count of any other trial. The highest density of *Planktothrix* in LHN trials only reached about 405,000. This is substantially lower than the density of BHN1

by eleven times. This large difference in results between the growth in bacteria without lettuce compared to that with lettuce when introduced to high nitrogen levels led to the expectation that these values would be significantly different. However, when the ANOVA test was run, there were only three replicates per trial. This low number of replicates ultimately led to an insignificant result, because the high density of BHN1 was not matched for BHN2 or BHN3. Those counts were lower near the counts of LHN1-3. This lack of replication in all trials was a large factor in what caused the results to be insignificant.

Although the tests determined each trial to be insignificantly different from one another, there is a general trend that can be seen. Overall, all trials without lettuce had higher density counts than those with lettuce, suggesting there is a possible connection to competition. This can be seen in Table 4, Table 5, and Graph 5. It is also seen in Plot 1 and Plot 2, where the results of B + L are lower than those of B. There is also a general trend that those trials that received high nitrogen and base phosphorus nutrients yielded higher density counts overall, suggesting there may be a link to *Planktothrix* growth and competition depending on the nutrient environment it grows in. Looking at Graph 3 and Graph 4, the rate of growth of *Planktothrix* tends to be faster in those trials that do not include lettuce than those that do include lettuce. This can suggest that lettuce slows the growth rate of *Planktothrix*. Similarly, looking at Graph 5, the average density counts of *Planktothrix* without lettuce when receiving high amounts of nitrogen towers over all other counts including those with lettuce, suggesting *Planktothrix* may thrive in environments of high nitrogen but only when not competing with lettuce. Overall, these suggestions cannot be deemed significant, though they can lead to further research where larger replications of each trial may lead them to be significant.

Errors: Due to limited resources and time, this project had areas that led to potential errors in results. The single light source above competing scenarios of lettuce v. non-lettuce may not have been enough light to evenly distribute among all trials. Of the samples containing lettuce, the light hit more directly above the LNP samples than it did LHP, CL, or LHP, with LHP the farthest from the light source. LHP lettuce plants began noticeably decaying by the second week of sample collections and were completely dead by the end of the run. By the last week, lettuce in LHN and LC began decaying as well, and LNP plants looked to be the most successful in growth and health (see figures 3-6 for lettuce progression). In observing the *Planktothrix*-only beakers, those closer to the edge of the counter seemed to have more success than those farther

back in the BHN and BHP samples. In BNP, the opposite occurred, where the sample towards the back of the counter had more production than those at the front, even though each sample in that category experienced identical nutrient addition. See figures 7-10 for *Planktothrix* progression. This could be the result of uneven lighting, where the angle the light hit at may have been stronger at the front of the counter in some samples and stronger at the back in other samples, leading to differing productivity in *Planktothrix*. Similarly, only one light source may not have been enough to grow the bacteria to their full potential; although *Planktothrix* is a low-light bacteria that does not require strong light sources, one light source still may have produced less bacteria than if there was a stronger, larger light to grow from (Halstvedt et al., 2007). The subjects also only received light from above for 24 hours a day the entire duration of the project. The rest of the room was kept dark for majority of the time, with exceptions every couple days for a couple hours at a time maximum. This irregularity in light penetration compared to normal daylight in nature may have affected overall growth in both test subjects.

Figure 7: Week 3 Planktothrix progress in BHN1-3. BHN1 has visibly more cell productivity than BHN2 or BHN3 and is positioned closer to the end of the table.

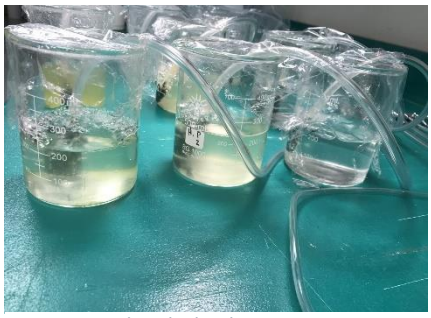


Figure 8: Week 3 Planktothrix progress in BHP1-3. BHP1-2 have visibly more Planktothrix productivity than BHP3 and are towards the front of the table.

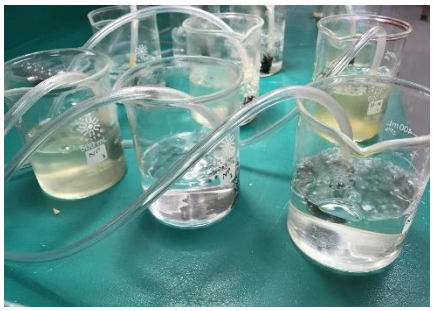


Figure 9: Week 3 Planktothrix progress in BNP1-3. BNP3 has visibly more Planktothrix productivity than BNP1-2 and is positioned furthest from the edge of the table.

Figure 10: Week 3 Planktothrix progress of all four conditions, showing variabilities in productivity within the columns.

Planktothrix is a slow growing bacterium when at low densities (Davis P.A. et al., 2002). Therefore, the very low starting density of 80,986 filaments/mL may have needed more than four weeks to grow a substantial number of bacteria from. The culture may have been too diluted to begin with, leading to lower count numbers and unrealistic results. Among the lettuce plants after the first week of sample collections, small unidentified flying insects started to appear. They were covering the tops of the leaves and were found in the water as well. These insects may have

affected the plants' abilities to absorb adequate light or grow to their full potential, thus affecting the growth the bacteria as well. See Figures 11-13 for insect visuals. With any research project,

Figure 11: Image of insects surrounding tabletop and top of lettuce leaves.

Figure 12: Image of insects covering dehydrated lettuce leaf.

Figure 13: Image containing insects on underside of leaf veins.

there also comes the error of human inaccuracy. There is the potential that not all filaments were visible under the microscope or a few filaments were counted twice. There is also the possibility that the bacteria were not distributed evenly throughout the water column before the samples were collected, despite best efforts. These errors were mitigated with the process of averaging the filament counts in the hopes that any outliers created by these errors were eliminated.

Conclusion:

The meaning behind the results and the inconclusiveness led me to fail rejection of the null hypothesis. If lettuce is introduced to a culture of *Planktothrix*, a competitive relationship is not formed between the two organisms. The presence of lettuce in the culture does not affect the growth and production of *Planktothrix*. This conclusion is relevant when determining ways to mitigate the production of algal blooms areas like the Sandusky Bay. As is mentioned above, *Planktothrix* is a cyanobacteria responsible in large for the production of these blooms, which can be damaging to the surrounding environment. Statistically, these results conclude that producing a system that connects the hydroponics of lettuce plants with the water growing *Planktothrix* will not mitigate the growth of the *Planktothrix*. However, with more replications of these trials, the removal of errors, and the efforts of continued research, a hydroponics system may be a solution to the problem these blooms pose, which could help limit the formation of algal blooms.

Future Research: If this project were to be done again with an unlimited amount of time, changes could be made to ensure more accurate results with less errors. First, the project would extend for more than four weeks to allow for optimum growth of the *Planktothrix*. If the project continues for fifty-two weeks, the *Planktothrix* numbers will grow exceedingly, either exponentially or at a limited amount where they will then decrease in numbers. Whatever the trend seen; more time allows for more information on how the *Planktothrix* grow with the lettuce. This would also require the use of a longer-living plant than lettuce or the continuous replacement of lettuce plants, as they only survive for about sixty days. Another change to the project would be the light sources. The light source used was not evenly distributed, changing the growth and decay rates of the lettuce plants as well as the *Planktothrix*. In replicating this project again, I would use a full spectrum grow light bar, spanning the length of the beaker distribution to ensure every sample receives the same intensity of light from all angles. There would be one light above the sources and one coming from beneath the beakers to ensure that there is enough light to reach the plants and the bacteria. These lights would be set on a timer with twenty hours receiving light and four hours of darkness to replicate more realistically what can occur in nature in the summer while also ensuring enough light is provided. Furthermore, I would begin with a denser culture of non-toxic *Planktothrix* so that it can grow easier in the experiment and also ensure that each beaker has a starting count. The low starting density of this project was risky in the possibility that no bacteria would survive for the first sample or future samples thereafter. Finally, at least ten replicates of each trial would be included, to ensure a more accurate ANOVA test. With these changes, the results of the project may better replicate real-life scenarios to help determine with more accuracy how an agricultural system would benefit from hydroponics of recycled nutrient water and how the algal blooms would be affected. With enough replications and changes to this initial project, eventually a consensus can be drawn on how agriculture can change for the better, if provided enough evidence.

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